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ORIGINAL ARTICLE

Predictive genetic markers of coagulation, inflammation and apoptosis in Perthes disease—Serbian experience

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Abstract Perthes disease is one of the most common forms of pediatric femoral head osteonecrosis with an unknown etiology. Coagulation factors were the first genetic factors suspected to have a role in the pathogenesis of this disease, but studies showed inconsistent results. It is described that inflammation is present during early stages of Perthes disease, but its genetic aspect has not been studied extensively. Little is known regarding the status of apoptotic factors during the repair process that leads to the occurrence of hip deformity in patients. Therefore, the aim of this study was to analyze major mediators involved in coagulation, inflammation, and apoptotic processes as possible causative factors of Perthes disease. The study cohort consisted of 37 patients. Gene variants of *TNF-α*, *FV*, *FII*, and *MTHFR* genes were determined by PCR-RFLP, while *IL-3* and *PAI-1* were genotyped by direct sequencing. The expression level of *Bax*, *Bcl-2*, *Bcl2L12*, *Fas* and *FasL* was analyzed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) technique. Our results

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showed a significantly increased level of expression of pro-apoptotic factor *Bax* along with significantly higher *Bax*/*Bcl-2* ratio in the patient group.

Conclusion: The results presented indicate that apoptosis could be one of the factors contributing to the lack of balanced bone remodeling process in Perthes patients.

What is Known:

- *The etiology of Perthes disease is unknown. The role of genetic factors involved in the coagulation process has been studied, showing inconsistent results so far.*
- *Genetic factors involved in inflammation and apoptotic processes that could contribute to development of hip deformity have not been studied extensively.*

What is New:

- *Our results show significantly increased level of expression of the pro-apoptotic factor Bax as well as significantly higher Bax/Bcl-2 ratios in patient group, indicating that apoptosis could be one of the factors contributing to the lack of a balanced bone remodeling process in Perthes patients.*

Keywords Blood coagulation factors · Bone remodeling · Inflammation · Apoptosis · Perthes disease

Abbreviations

BAX	Bcl-2-associated X protein
BCL-2	B cell lymphoma 2
BCL2L12	BCL2-like 12
FAS	Fas receptor
FASL	Fas ligand
FV	Factor V Leiden
FII	Factor II
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL-3	Interleukin-3
LCPD	Legg–Calve–Perthes' disease
MTHFR	Methylenetetrahydrofolate reductase
PAI-1	Plasminogen activator inhibitor-1
PBMNC	Peripheral blood mononuclear cells
TNF-α	Tumor necrosis factor alpha

Introduction

Legg–Calve–Perthes' disease (LCPD; Perthes disease) is an idiopathic osteonecrosis of the immature, developing femoral head [21, 35]. It is a rare disease with a great variation of annual incidence, from 1/250,000 in Hong Kong and 1/18,000 in the UK to 1/3,500 in the Faroe Islands (www.orpha.net). It occurs approximately five times more commonly in boys than in girls [31].

Despite nearly 100 years of detailed characterization of its clinical and radiological features, as well as a research devoted to the pathophysiology of this disease, its etiology remains essentially unknown [26]. Various possible causes have been proposed, including repetitive microtrauma, skeletal retardation and vascular insufficiency [17, 26]. The prevailing view is that both environmental and genetic factors contribute in varying degrees to the onset and development of Perthes disease [14].

It has been shown in experimental models that disrupted blood supply and infarction of the femoral head cause changes similar to those in Perthes patients [19]. Genes related to the coagulation process were the first genetic factors suspected to have a role in the pathogenesis of Perthes disease, causing the impaired local circulation. Previous studies related to the role of coagulation factors in the etiology of Perthes disease provided controversial results, as described in recent meta-analysis [37].

It is well documented that inflammatory cytokines inhibit bone formation and stimulate resorption [9, 20, 36]. There are four well-accepted stages of the pathogenesis of Perthes disease: synovitis, avascular (condensation), fragmentation (resorption) and residual (healed) phase [18, 21]. Inflammation is present during the development of the disease, and inflammatory changes are most marked during the fragmentation phase [7]. We have recently shown that the *IL-6* promoter gene variant, which influences expression of the *IL-6* gene [11], is associated with Perthes disease [34]. *TNF-α* has been recognized as a skeletal catabolic agent that stimulates osteoclastogenesis while simultaneously inhibiting osteoblast function [25]. This cytokine is crucial to the pathogenesis of the bone and joint destructions that occur in various inflammatory and rheumatic diseases [10, 22, 24]. Interleukin-3 has important functions with regard to bone turnover [40], and gene variants in *IL-3* were shown to be associated with rheumatoid arthritis, Graves' disease, asthma and atopy [5, 29, 39].

It is known that apoptosis of osteoblasts and osteoclasts is strictly regulated and plays an important role in both the maintenance of physiologic bone turnover and in the development of pathological skeletal conditions [23], but none of the studies on Perthes disease have questioned this mechanism so far. One of the most important factors in the pathophysiology of Perthes disease, which greatly contributes to the development of severe deformity of the affected hip, is an imbalance in bone remodeling, which takes place during the fragmentation phase [16]. Impaired apoptosis is one of the factors that could affect the repair process, which takes place during the fragmentation phase as well as during the avascular phase that precedes it. The inner apoptotic pathway is regulated by the BCL-2 family of proteins, in which the balance between pro-apoptotic and anti-apoptotic molecules is the main determinant of the apoptotic status of the cell. It has been shown that an increased BAX/BCL-2 ratio accelerates the apoptotic process [27]. The *Bcl2L12* gene is a new member of the BCL-2 family, with anti- and pro- apoptotic function [33]. The extrinsic apoptotic pathway, where the FAS/FASL system plays a

crucial role, is involved in the regulation of bone turnover and may represent a critical link between the immune system and bone remodeling in physiological and pathological processes [38].

Here we analyzed the association of genetic variants of genes involved in coagulation, *FV*, *FII*, *MTHFR* and *PAI-1*, with Perthes disease. Also, we have examined genetic variants of inflammatory mediators relevant for bone remodeling, *TNF-α* and *IL-3* genes, as susceptibility factors for Perthes disease. In order to address the question about the role of apoptotic mechanism in the formation of hip deformity in Perthes disease, we examined the expression pattern of main apoptotic factors, *Bax*, *Bcl-2*, *Bcl2L12*, *Fas* and *FasL*, in our patient group.

Materials and methods

Patients and control subjects

Our study was approved by the Ethics Committee of the Institute for Orthopedic Surgery "Banjica," Belgrade, Serbia. Written informed consent was obtained for all patients.

This study enrolled 37 patients, 29 males and 8 females with a mean age of 9.10 years (SD—5.31). For all 37 patients, DNA analyses of gene variants involved in coagulation and inflammation processes were performed. As a control, for the comparison of gene variant frequencies for *PAI-1*, *TNF-α*, and *IL-3* genes, a group of 50 healthy donors was used. This control group was sex matched and included 35 male and 15 female voluntary donors, with a mean age of 29.92 years (SD—12.74). For *FV* Leiden, *FII*, and *MTHFR* genes, a new control group was used, which included another 100 healthy voluntary donors, 46 male and 54 female, with a mean age of 9.5 years (SD—0.5) [8].

For the analysis of mRNA expression, samples of 31 patients (out of 37) were used, since RNA samples were not available for 6 of them. This group consisted of 25 males and 6 females, with a mean age of 7.52 years (SD—2.83). An additional healthy control group was included for expression analysis. It consisted of 11 voluntary donors, 6 males and 5 females with a mean age of 6.39 years (SD—3.86).

All patients were diagnosed with Perthes disease between 1998 and 2012, at the Institute for Orthopedic Surgery "Banjica," Belgrade. The diagnosis was established according to standard clinical criteria (onset of groin pain, disturbed stance on the affected leg and waddling gait, limitation of hip joint movements, especially internal rotation, absence of clinical signs suggesting trauma or infection), ultrasonographic examination (verification of homogenous hip joint effusion), and radiographic signs (condensation or fragmentation of the epiphyseal ossification center, loss of femoral head sphericity). Two patients had bilateral disease.

Blood sampling, DNA, RNA and cDNA preparation

Venous blood was collected into two 4.5-ml sodium citrate anticoagulant tubes (Vacutainer, Becton-Dickinson, Plymouth, UK) and stored at +4 °C until processing. Within 4 h after sampling, 1 ml of whole blood was separated for DNA isolation and stored at −20 °C, while the rest of the sample was used for peripheral blood mononuclear cell (PBMNC) isolation by Ficoll-Histopaque density gradient (GE Healthcare, Sweden).

Genomic DNA was isolated from whole peripheral blood with QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) and stored at −20 °C. The isolated PBMNC were stored at −80 °C in TRI Reagent solution (Ambion, USA) until the RNA extraction was done, according to the manufacturer protocol. Complementary DNA (cDNA) was prepared from 1 μg of RNA using RevertAid Reverse Transcriptase (Thermo Scientific, USA) and random hexamer primers.

Detection of coagulation- and inflammation-related gene variants

The detection of gene variants of coagulation factors *FV* 1691G>A (rs6025), *FII* 20210G>A (rs1799963), and *MTHFR* 677C>T (rs1801133) was performed using a PCR-RFLP method described elsewhere [8]. *PAI-1* 4G/5G (rs1799889) gene variant was genotyped by direct sequencing using primers described elsewhere [15]. The PCR products were directly sequenced using the BigDye Terminator Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), and sequencing was performed on ABI PRISM 310 Genetic Analyzer using Sequencing Analysis Software (Applied Biosystems). Gene variant *TNF-α*-308G>A (rs1800629) was detected by PCR-RFLP technique, while *IL-3*-16C>T (rs31480) and C132T (rs40401) gene variants were detected by direct sequencing (details included in Supplementary material).

Relative quantification of apoptosis-related gene expression

All analyzed genes, primers, probe and assays used for the quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) are shown in Supplementary Table 1.

qRT-PCR analyses for all analyzed genes were performed on 7500 Real-Time PCR System (Applied Biosystems). Melting curve analyses was performed for reactions in which SYBR Green chemistry was used, to ensure the specificity of the products. In all qRT-PCR experiments, the *GAPDH* gene was used as the endogenous control, in order to normalize the obtained results. All expression analyses were performed in

duplicate. In the case of discordant results, the analyses were repeated in duplicate. Relative quantification analyses were performed by a comparative ddCt method, using a median value of normalized target gene expression level of the healthy control group as the calibrator.

The expression of *Bax*, *Bcl-2*, *Bcl2L12* and *GAPDH* genes was analyzed by qRT-PCR using KAPA SYBR FAST Universal 2× qPCR Master Mix (KAPA Biosystems, USA) with primers that were taken from listed publications [1, 12, 28]. *Fas* and *FasL* expression levels were determined by qRT-PCR using the TaqMan Gene Expression Assays and KAPA PROBE FAST Universal 2× qPCR Master Mix (KAPA Biosystems, USA). To normalize expression of these genes, *GAPDH* expression level was determined using primers and probes described elsewhere [6].

Statistical analysis

The differences in genotype and allele distributions between patient and control groups were analyzed by Fisher's exact test. The consistency of genotype distributions with Hardy–Weinberg equilibrium was tested using Pearson chi-square test, for each cohort. Differences in expression levels were analyzed using the Mann-Whitney *U* test. All tests were two-sided, and differences were considered to be significant in all cases when $P<0.05$. Data was analyzed using the SPSS for Windows 20.0 software (SPSS, Inc, Chicago, IL, USA), and the power of the test was performed using the calculator from http://powerandsamplesize.com. Linkage disequilibrium values (D', r^2) between gene variants were tested using Haploview software, Version 4.2.

Results

Contribution of genetic markers involved in coagulation and inflammation processes in Perthes disease

Coagulation factors FV, FII, MTHFR and PAI-1

The results of the analysis of the *FV*, *FII*, *MTHFR* and *PAI-1* gene variants in patients with Legg–Calve–Perthes disease and controls are shown in Table 1. All genotype distributions were in Hardy–Weinberg equilibrium. When genotype and

Table 1 Genotype and allele distributions of coagulation factors among Perthes patients and controls

Gene variant	Genotype	Patients *n*=37 (frequency)	Controls *n*=100 (frequency)	OR (95 % CI)	*P* Value
FV Leiden	GG	35 (0.94)	92 (0.92)	0.65 (0.13–3.24)	0.72
G1691A	GA	2 (0.60)	8 (0.80)		
	AA	0	0		
Allele frequency	G	0.97	0.96	0.67 (0.13–3.21)	0.73
	A	0.03	0.04		
GG vs GA+AA					
FII	GG	35 (0.94)	99 (0.99)	5.65 (0.49–64.33)	0.17
G20210A	GA	2 (0.60)	1 (0.01)		
	AA	0	0		
Allele frequency	G	0.97	0.99	2.75 (0.38–19.88)	0.57
	A	0.03	0.01		
GG vs GA+AA					
MTHFR	CC	19 (0.51)	33 (0.33)	0.46 (0.21–1.00)	0.07
677C>T	CT	12 (0.32)	54 (0.54)		
	TT	6 (0.16)	13 (0.13)		
Allele frequency	C	0.68	0.60	0.72 (0.41–1.26)	0.26
	T	0.32	0.40		
CC vs CT+TT					

Gene variant	Genotype	Perthes patients *n*=37	Controls *n*=50	OR (95 % CI)	*P* Value
PAI-1 4G/5G	5G/5G	9 (0.24)	8 (0.16)	0.45 (0.16–1.31)	0.20
	4G/5G	22 (0.59)	27 (0.54)		
	4G/4G	6 (0.16)	15 (0.30)		
Allele frequency	5G	0.54	0.68	0.64 (0.35–1.17)	0.17
	4G	0.46	0.43		
5G/5G+4G/5G vs 4G/4G					

OR odds ratio, *CI* confidence interval

allele frequencies were compared among patient and control groups, no significant differences were observed. For *FV* Leiden and *FII* G20210A, we did not find any homozygote carriers either in patients or in control group.

Inflammation factors IL-3 and TNF-α

A perfect linkage disequilibrium was observed between the C-16T and C132T polymorphisms in *IL-3* gene ($D'=1.0$, $r^2=1.0$). Therefore, *IL-3* C-16T and C132T genotypes and alleles occurred with identical frequencies. The comparison of genotype and allele frequencies among patients and controls did not reveal significant differences neither for *TNF-α* nor for *IL-3* analyzed gene variants (Table 2).

Expression of apoptotic factors in Perthes patients

Stratification of patients for expression analyses

The thirty-one patients used for expression analyses (further designated as total patient group) were sampled in different phases of the disease: 6 patients in avascular, 14 in fragmentation, 8 in reossification and 3 in residual phase. All patients were sampled only once within an indicated phase of the disease. As mentioned before, one of the most important factors in the pathophysiology of Perthes disease is the imbalance between resorption and bone anabolism, which takes place during the fragmentation phase of the disease. Our assumption was that an impaired apoptotic process, in particular during the fragmentation phase as well as during the avascular phase which precedes it, could contribute to the lack of new bone formation. Therefore, in order to test that hypothesis, a subgroup of patients that were sampled in the avascular and fragmentation phases (designated as A+F patient group) was formed and used in analyses alongside the total patient group. The A+F patient subgroup consisted of 15 males and 5 females with a mean age of 6.10 (SD—1.69).

Expression of inner apoptotic pathway genes Bax, Bcl-2 and Bcl2L12 in Perthes patients

To determine the expression pattern of inner apoptotic pathway-related genes in Perthes disease, we detected the expression levels of *Bax*, *Bcl-2* and *Bcl2L12* genes in PBMNC of patient and control groups. There was no significant difference in the *Bax* expression level between the total patient and control groups (Fig. 1a), but the *Bax* expression level was significantly increased in the A+F patient subgroup in comparison to the control group ($P=0.023$, Fig. 1a). No significant difference in the expression level of *Bcl-2* or *Bcl2L12* genes was detected (Fig. 1b, c). Since it has been suggested that the ratio of *Bax* and *Bcl-2*, rather than the level of either gene, is predictive of cell fate [27], we also determined the *Bax*/*Bcl-2* ratio. Our results showed that the *Bax*/*Bcl-2* ratio was significantly higher in the total patient group compared to the control group ($P=0.047$, Fig. 1d). In addition, the *Bax*/*Bcl-2* ratio was significantly higher in A+F patient subgroup in comparison to the control group ($P=0.032$, Fig. 1d).

Expression of extrinsic apoptotic pathway genes Fas and FasL in Perthes patients

Next, we evaluated the expression level of genes involved in extrinsic apoptotic pathway, *Fas* and *FasL*, in the PBMNC of patient and control groups. Comparison of the total patient group and A+F subgroup to the control group showed no significant differences in the expression of *Fas* or *FasL* genes (Fig. 1e, f).

Table 2 Genotype and allele distributions of inflammation-related genes among Perthes patients and controls

Gene variant	Genotype	Patients $n=37$ (frequency)	Controls $n=50$ (frequency)	OR (95 % CI)	*P* Value
TNF-α	GG	23 (0.62)	38 (0.76)	1.93 (0.76–4.88)	0.24
G-308A	GA	13 (0.35)	11 (0.22)		
	AA	1 (0.03)	1 (0.02)		
Allele frequency	G	0.80	0.87	1.67 (0.78–3.58)	0.25
	A	0.20	0.13		
GG vs GA+AA					
IL-3	CC/CC	18 (48.6)	25 (50.0)	1.06 (0.45–2.47)	1
C-16T/C132T	CT/CT	17 (45.9)	24 (48.0)		
	TT/TT	2 (5.4)	1 (0.02)		
Allele frequency	C/C	0.72	0.74	1.11 (0.59–2.07)	0.87
	T/T	0.28	0.26		
CC vs CT+TT					

OR odds ratio, *CI* confidence interval

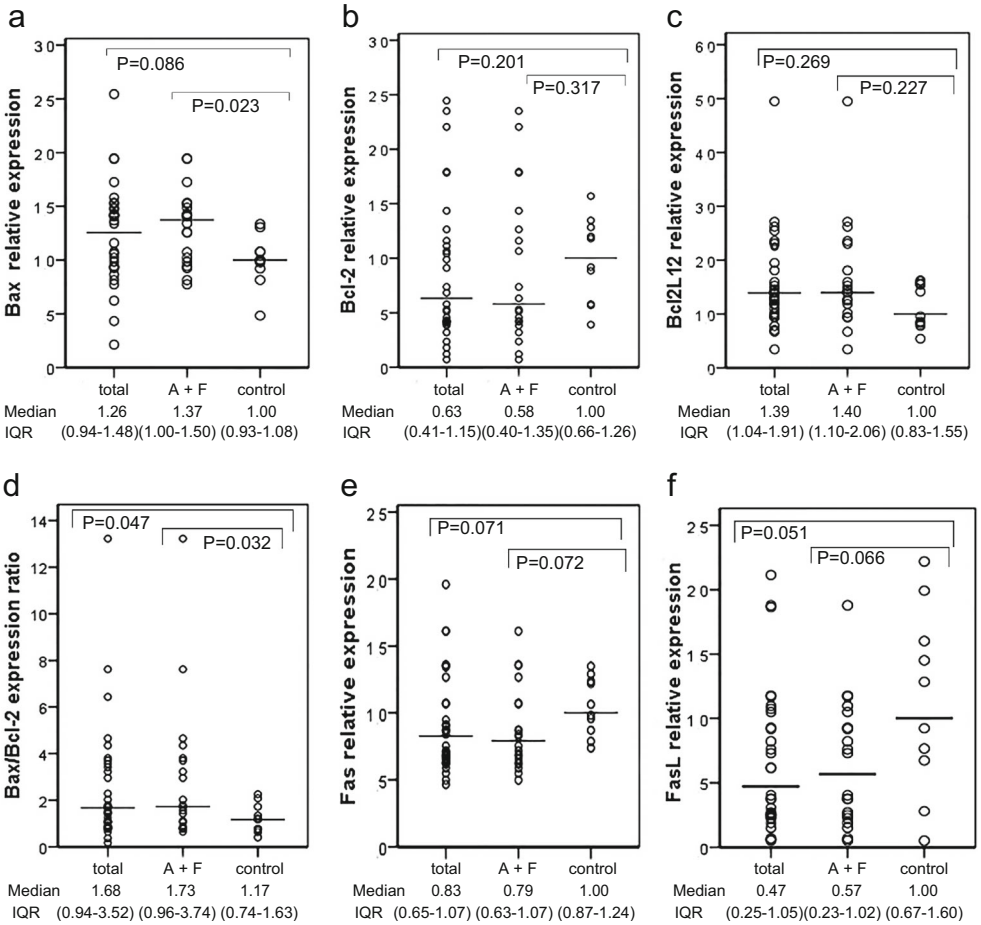

Fig. 1 Relative expression of genes of intrinsic and extrinsic apoptotic pathways in Perthes patients and controls. **a** *Bax*, **b** *Bcl-2*, **c** *Bcl2L12*, **d** *Bax*/*Bcl2* ratio, **e** *Fas*, **f** *FasL. Total* represents total patient group, *A+F* the subgroup of patients sampled in avascular and fragmentation phases, and *IQR* represents the interquartile range. The *line bars* represent the median value (50th percentile) for each cohort. *P* values were calculated by Mann–Whitney *U* test

Discussion

Many studies have been performed with the aim of explaining the nature of Perthes disease, but it still remains one of the most controversial conditions in pediatric orthopedics [17, 30]. The focus of our study was to examine genetic factors, such as gene variants in coagulation- and inflammation-related genes, as well as the gene expression profiles of the main regulators of intrinsic and extrinsic apoptotic pathways, as possible causative factors involved in the pathogenesis of Perthes disease.

The role of genetic factors involved in the coagulation process that could contribute to impaired local circulation has been extensively studied. Recent meta-analysis has summarized conflicting results from previous studies on gene variants in coagulation factors *FV*, *FII*, and *MTHFR* and Perthes disease [37]. It showed that *FV* might increase the odds of developing Perthes disease about threefold. In our cohort of patients, we did not find significant association of gene variants in coagulation factors with Perthes disease.

Although studies on inflammatory cytokines in different bone and cartilage diseases have been conducted [9, 20, 36], this is the first report regarding the gene variants of inflammatory mediators *TNF-α* and *IL-3* in Perthes patients. We found no significant difference between the frequencies of analyzed gene variants in *TNF-α* or *IL-3* genes between the patient and control groups. In our study group, the C132T variant in the *IL-3* gene was in perfect linkage disequilibrium with the promoter polymorphism *IL-3* C-16T. It is interesting to note that the frequency of −16C/132C allele of the *IL-3* gene was rather high in our patient and control group, pointing to the population specificity of frequency of this allele. This allele frequency in our population was similar with other Caucasian subjects [13], while the studies from Asia reported much lower frequency in their populations [5, 29, 39].

This is the first report on the status of the main apoptotic factors in Perthes patients. Research on osteonecrosis of the femoral head has shown that the process leading to cell death in the femoral head of such patients includes an increased rate of apoptosis rather than purely the necrosis of bone cells [3]. Additionally, apoptotic bodies and DNA fragmentations have been observed in the osteocytes and marrow cells of the ischemically necrotic femoral heads of rats [2].

Skeletal tissue integrity is a result of the delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts [23]. In addition to osteoclasts, a distinct

population of resident macrophage cells, OsteoMacs, has been shown to be an integral component of bone tissue and to play an important role in bone homeostasis [32]. These cells constitute approximately one sixth of the total cells within osteal tissues [4]; they are intercalated with bone lining cells and promote osteoblast matrix production and/or mineral deposition by secreting a wide range of osteo-active factors [32]. Additionally, it has been shown that the systemic or local depletion of macrophages delayed bone healing and impaired bone formation, thus showing that OsteoMacs have a role in bone modeling and remodeling [4].

Our study points to the relevance of the inner apoptotic pathway in the pathophysiology of Perthes disease. More precisely, we found a significantly increased expression level of the pro-apoptotic factor *Bax* in the PBMNC of the subgroup of patients that were sampled in the avascular and fragmentation phases of the disease. In addition, the *Bax*/*Bcl-2* ratio was significantly higher in both the total patient group and in a subgroup of patients that were sampled in the avascular and fragmentation phases of the disease, in comparison to the control group. We hypothesized that this distorted apoptotic process may contribute to the pathological remodeling of the affected femoral head through the significant decrease in the number of resident osteal macrophages, OsteoMacs.

Our study has a number of strengths and some limitations. This is the first report showing increased expression level of a pro-apoptotic factor, implicating the role of apoptotic process in the pathogenesis of Perthes disease. The limitation of the present work is the relatively small sample size and control group design. Since Perthes disease is a rare disease with a limited number of patients diagnosed in Serbia, a relatively small group of patients was studied. This resulted in the low power of the test for associative study (<0.47), but the number of patients included in the expression analysis is quite relevant. Also, different control groups were used in this study. For coagulation and inflammation factors, we used the same control groups as in previous studies of these factors in Serbia [8, 34]. A new control group was designed for the expression study of apoptotic factors.

In conclusion, our study brings new insights into the role of genetic factors in the pathophysiology of Perthes disease. If further studies on larger patient cohorts confirm that apoptosis is disturbed in Perthes patients, apoptotic factors may become reliable biomarkers for the evaluation of the status or the prognosis of the disease, as well as a potential target for therapy.

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Ethical statements The study was approved by the Ethics Committee of the Institute for Orthopedic Surgery "Banjica," Belgrade, Serbia (I-86/34; 7-2, 12. 12. 2012.). Written informed consent was obtained for all patients.

Conflict of interest The authors declare that they have no conflict of interest.

Author's Contributions DS, ZB and ZŽ performed the case and control sample collection and clinical management of patients. ZTŠ and DM participated in clinical management of patients and participated in studz design. VDJ provided control sample for coagulation study and participated in data analyzes. SS and VS performed the laboratory work and statistical analysis. SS and GN analyzed data and wrote first draft of the paper. VS and SP designed the study and wrote the final version of the paper. VS take the primary responsibility for the paper.

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